PROTECTION AGAINST THE VESICANT CHEMICAL WARFARE AGENT SULFUR MUSTARD: THERAPEUTICS UTILIZING APOPTOSIS INHIBITORS

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ABSTRACT

Sulfur mustard (SM, bis-(2-chloroethyl) sulfide), commonly called mustard gas, is a vesicant chemical warfare agent and a potential terrorism agent. SM is relatively easy to make and to deploy, which makes this chemical most likely to be used. SM exposure causes debilitating skin blisters (vesication) and injury to the eyes and the respiratory tract. Therefore, developing an effective medical countermeasure to protect against the dermal, ocular and airway injuries due to this dreaded chemical agent is an urgent priority of the US Army. SM pathophysiology is consistent with epithelial cell damage, particularly basal cell apoptosis. SM-induced apoptosis may occur via multiple pathways dependent on one or more of the following: (a) abnormal Ca^{2+} homeostasis, (b) disturbed cellular bioenergetics, and (c) Fas (death receptor) response. Apoptosis pathways are characterized by the involvement of the pathway-specific caspases (cysteine aspartase). We determined caspase activity by assay of fluorogenic caspase type-specific peptide substrate hydrolysis. We studied caspase processing, i.e., proteolytic conversion of procaspase to active caspase by immunoblot analyses utilizing caspase type-specific antibodies. Our results in cell culture models of both human epidermal keratinocytes and human airway epithelial cells indicated that SM activates (a) caspase-9, an indicator of the Ca^{2+}/CaM-mediated mitochondrial pathway, (b) caspase-8, a marker for the Fas-mediated pathway, and (c) caspase-3, the executioner caspase involved in both pathways. A peptide caspase inhibitor, specific for caspase-3 (AC-DEVD-CHO), added to cells prior to SM decreased apoptosis. These observations suggest apoptosis as a mechanism of SM toxicity and caspase inhibitors as prospective medical countermeasures.

1. INTRODUCTION

SM (mustard gas) is both a chemical warfare agent and a terrorism agent. SM causes severe injury to the skin (blisters), the eyes, and the respiratory tract (Papirmeister *et al*., 1991). SM pathophysiology is consistent with epithelial cell damage, particularly basal cell apoptosis, i.e., programmed cell death (Dabrowska *et al*., 1996, Rosenthal *et al*., 1998, Ray *et al*., 2002). SM-induced apoptosis may occur via multiple pathways dependent on one or more of the following: (a) abnormal Ca^{2+} homeostasis, (b) disturbed cellular bioenergetics, and (c) Fas (death receptor) response. Apoptosis pathways are characterized by the involvement of the pathway-specific caspases (cysteine aspartase) (Keppler-Hafkemeyer *et al*., 1998; Kumar, 1999; Porter and Janick, 199). The Ca^{2+}/calmodulin (CaM)-dependent pathway involves the initiator caspase-9 and then the executioner caspase-3. The Fas-mediated pathway involves caspase-8 and caspase-3. We characterized the SM-induced apoptosis pathways in primary human keratinocytes (HEK) and airway epithelial cells (HAEC) by studying (a) caspase activity (fluorogenic substrate hydrolysis) and (b) caspase processing (immunoblot analyses). The results show that in both HEK and HAEC, SM stimulates caspase-9, caspase-8 and caspase-3; caspase-3 is sensitive to a peptide caspase inhibitor.

2. MATERIALS AND METHODS

2.1 Cells and Chemicals

Frozen stock normal human epidermal keratinocytes (HEK), bronchial/tracheal epithelial cells (NHBEC), and small airway epithelial cells (SAEC) and growth media (KGM, BEGM, SAGM) were obtained from CAMBREX, Walkersville, MD. SM (>98% pure) was obtained from the US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA.

The caspase-3 assay kit containing the fluorogenic cell permeable caspase-3 substrate, Z-DEVD-R110 ((bis-(N-CBZ)-asp-glu-val-asp-amide-rhodamine 110) was obtained from Beckman-Coulter, Fullerton, CA (Product code: Beckman-Coulter CellProbe™ HT Caspase-3 Whole Cell Assay Kit). Other substrates for caspase-3 (cell impermeable AC-DEVD-AMC (acetyl-asp-glu-val-asp-7-amido-4-methylcoumarin)), caspase-8 (AC-IETD-AMC (acetyl-Iso-leu-glu-thr-asp-7-amido-4-

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methylcoumarin)) and caspase-9 (Ac-LEHD-AMC (acetyl-leu-glu-his-asp-7-amido-4-methylcoumarin)) were obtained from Sigma-Aldrich, Saint Louis, MO. The reversible caspase-3 inhibitor, AC-DEVD-CHO (aldehyde) and the irreversible general caspase inhibitor, Z-VAD-FMK (benzyl oxycarbonyl-val-ala-asp-fluoromethylketone) were obtained from BD Biosciences, San Jose, CA. All other chemicals were of the purest grade available.

2.2 Cell Culture

Frozen stock cells (second passage, 5 x 10^5 cells/vial) were cultured in either 75 or 150 cm^2 tissue culture flasks (10^5 to 3 x 10^5 cells/flask) in respective growth medium containing supplementations as applicable according to company instructions to initiate the culture. When the monolayer cells became 70-80% confluent, they were sub-cultured to 80-90% confluency in 75 or 150 sq. cm. flasks or in multi-well plates (96- or 24-well) and used in the experiments. To maintain consistency, cells from a single donor and sub-cultured only to passage 3 were used.

2.3 Exposure of Cells to SM

Cells were exposed to diluted SM in growth medium using a unique formulation developed by Broomfield and Gross (1989) and as described by Ray *et al.* (1995) for specified concentrations and times. Briefly, the formulation consists of a 10 µl neat SM oily globule frozen in 10 ml of respective growth medium. This formulation is thawed by warming to room temperature and vortexed at top speed for 1 min to solubilize SM in the medium to produce a 4 mM stock solution. The stock solution is then added to the liquid medium in cell culture vessels for desired SM concentrations.

2.4 Caspase-3 Assay

The caspase-3 fluorometric assays were done using either cell extracts (Simbulan-Rosenthal *et al.*, 2002) or microplate (96-wells) monolayer cultures (Ray *et al.*, 2005). The substrates used for assays using cell extracts and monolayer cultures were AC-DEVD-AMC and Z-DEVD-R110, respectively. The assay was based on the hydrolysis of the caspase-3 peptide substrate conjugated to a fluorochrome resulting in the release of the fluorescent AMC or R110 moiety. The fluorescence (absolute units) was measured using the PerSeptive Biosystems CytoFluor^R Multi-Well Plate Reader Series 4000 spectrofluorometer.

2.5 Caspase-8 and Caspase-9 Assays

Caspase-8 and caspase-9 assays were done based on the same principle of fluorogenic peptide caspase substrate hydrolysis using AC-IETD-AMC and AC-LEHD-AMC, respectively. However, extracts from monolayer cells grown in 150 sq. cm. were used instead of microplate (96-wells) monolayer cultures; the microplate method did not generate adequate signals.

2.6 Immunoblot analysis

SDS-PAGE and transfer of separated proteins to nitrocellulose membranes were performed according to standard protocols (Rosenthal *et al.*, 1998, 2003). Proteins were measured (DCA protein assay; BioRad, Hercules, CA) and Ponceau S (0.1%) staining of membranes was performed to verify equal loading and transfer of proteins. Membranes were then incubated with antibodies to the p17 subunit of caspase-3 (1:200; Santa Cruz Biotech, Santa Cruz, CA), procaspase-8 (1:1000; PharMingen, San Hose, CA), or procaspase-9 (1:1000; Cell Signaling, Danvers, MA). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase–conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce, Rockford, IL). Immunoblots were sequentially stripped of antibodies and reprobed with additional antibodies to compare different proteins from the same filter.

3. RESULTS AND DISCUSSION

3.1 Apoptosis Pathways

A schematic representation of the possible pathways of apoptosis due to SM exposure in cultured cells is shown in Figure 1. SM may cause apoptosis via different pathways involving (a) Ca2+-calmodulin (CaM), (b) death (Fas) receptors, (c) endoplasmic reticulum (ER) Ca2+, and (d) energy depletion (Ray *et al.*, 2002). In apoptosis, the caspases play a crucial role and the caspase types involved differentiate apoptotic pathways (Gill *et al.*, 2002). The Ca2+-CaM and the energy depletion pathways activate caspase-9, which then activates caspase-3, the executioner caspase responsible for the apoptotic end points. The Fas receptor pathway activates caspase-8 and then caspase-3. The (ER)-Ca2+ pathway may activate either a Ca2+-stimulated cystein protease calpain, which may then activate caspase-3, or directly caspase-12 that causes apoptosis.

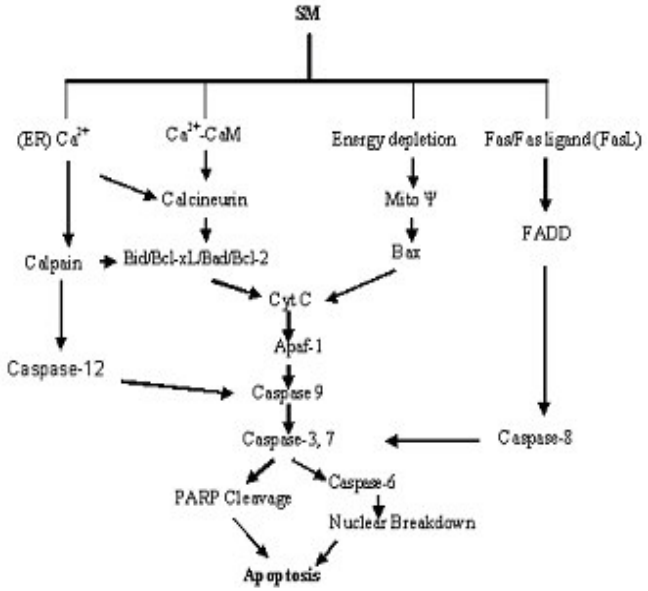

FIGURE 1. Hypothetical representation of SM-induced apoptosis pathways

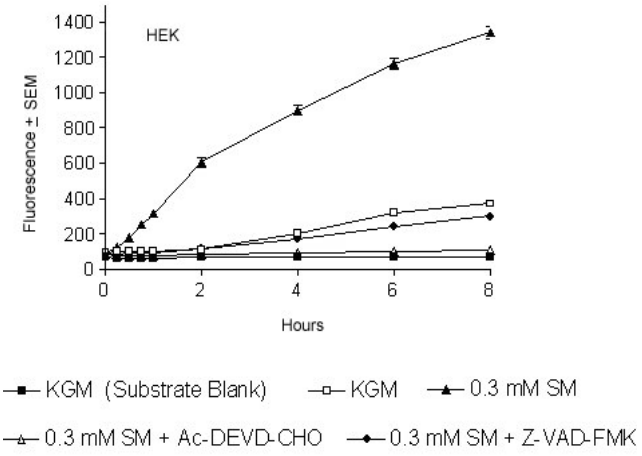

FIGURE 2. SM-induced Apoptosis in HEK. The results shown as absolute fluorescence units are mean ± SEM of eight replicate determinations. The absence of SEM bars means that the values are too small to be visible. Significant differences ($p < 0.01$) were observed between cells exposed to 0.3 mM SM vs. other groups.

3.2 SM Causes Apoptosis in Cultured Human Epidermal Keratinocytes (HEK)

In HEK, SM stimulates caspase-3 (apoptosis), which is inhibited by peptide caspase inhibitors. HEK cultures in KGM in 96-well plates were first incubated inside a cell culture incubator without or with the caspase-3 specific inhibitor AC-DEVD-CHO (0.1 mM) or the general caspase inhibitor Z-VAD-FMK (0.01 mM) for 30 minutes prior to 0.3 mM SM exposure. The cells were then incubated overnight and caspase-3 assays were conducted as described in Materials and Methods. The time intervals (hours) are after the caspase-3 substrate (Z-DEVD-R110) addition to cells following SM exposure for about 16-18 hours. The results shown in Figure 2 indicate that 0.3 mM SM, which is considered to be the in vitro equivalent of a vesicating (skin blistering) concentration in vivo, causes caspase-3 activation, i.e., apoptosis in HEK. This SM-induced apoptosis is effectively prevented by addition of the caspase-3 specific peptide inhibitor AC-DEVD-CHO or a general caspase inhibitor Z-VAD-FMK to the cell culture medium prior to SM exposure.

3.3 SM Causes Apoptosis in Cultured Human Airway Epithelial Cells (HAEC)

We examined whether SM stimulates caspase-3 in HAEC using the same microplate monolayer culture assay used for HEK. Results (Figure 3) showed that as in HEK, 0.3 mM SM stimulates caspase-3 (apoptosis) in both normal human bronchial/tracheal epithelial cells (NHBEC, upper respiratory tract model) and small airway epithelial cells (SAEC, deep lung model). We also tested the effect of the peptide caspase-3 inhibitor, AC-DEVD-CHO on SM-induced apoptosis in NHBEC. Like HEK, apoptosis due to SM is inhibited by AC-DEVD-CHO in NHBEC (Figure 4). These results taken together (Figures 2, 3 and 4) suggest that apoptosis is one of the mechanisms of toxicity in both skin and the airways and a peptide caspase inhibitor may be a prospective medical countermeasure against vesicant action.

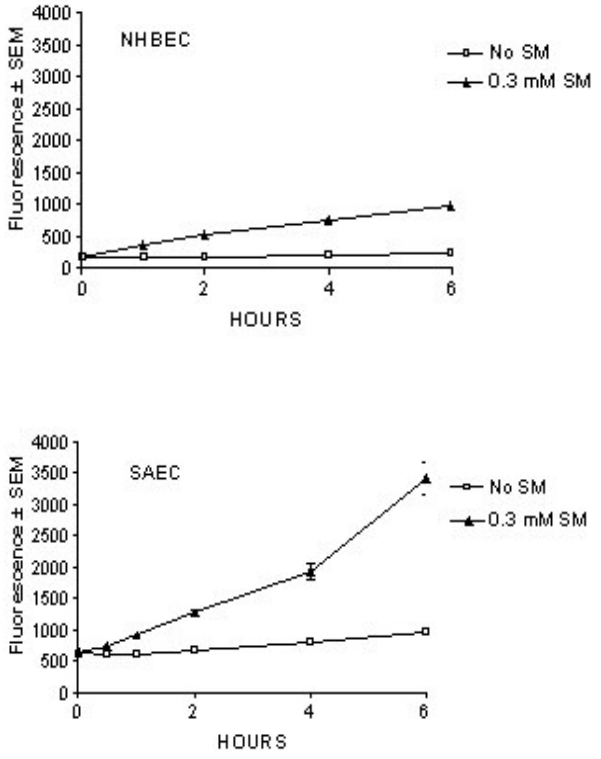

FIGURE 3. SM-induced Apoptosis in NHBEC and SAEC. Other details are similar to HEK experiments in Figure 2.

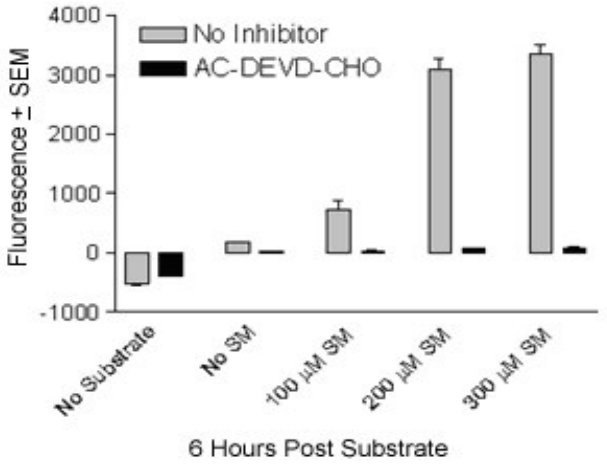

FIGURE 4. The caspase-3 inhibitor AC-DEVD-CHO prevents SM stimulation of caspase-3 in NHBEC. Other details are as in Figure 2.

3.4 Mechanisms of Apoptosis Due to SM in the Skin and Airways

Previously, we published results (Rosenthal *et al.*, 2003) indicating that in HEK, SM causes apoptosis via at least two pathways: (a) intrinsic mitochondrial pathway involving the activation of caspase-9 followed by caspase-3, and (b) extrinsic Fas (death receptor) mediated pathway involving caspase-8 and then caspase-3. In this report, we also demonstrated that expression of dominant-negative Fas-associated death domain (FADD-DN) blocks human keratinocyte apoptosis and vesication induced by SM in a human skin grafted on nude mouse model. We, therefore, became interested in studying the mechanisms of apoptosis due to SM in airway epithelial cells. In these studies, we utilized SM (0.3 mM)-exposed cell extracts to examine apoptosis pathways by measuring caspase-9, caspase-8 and caspase-3 by fluorometric caspase substrate hydrolysis assays as well as by Western blotting analyses of caspase processing as described in Materials and Methods. Our results presented in Figures 5 and 6 show that the mitochondrial and Fas mediated mechanisms of apoptosis due to SM are common features in the airways (SAEC) as also seen in the skin (HEK). Similar results were obtained with NHBEC (data not shown). Based on these observations, we propose apoptosis inhibitors may have potential applications as therapeutics against the vesicant chemical warfare agent SM.

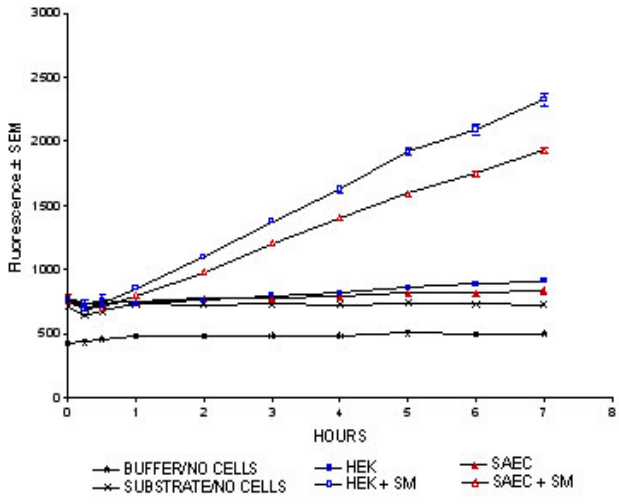

FIGURE 5. SM (0.3 mM) activates caspase-8 in cultured HEK and SAEC. The caspase-8 substrate was AC-IETD-AMC. Assays were done using extracts from monolayer cells grown in 150 sq. cm. flasks as described in Materials and Methods. SM exposure and other experimental details are similar to HEK experiment in Figure 2.

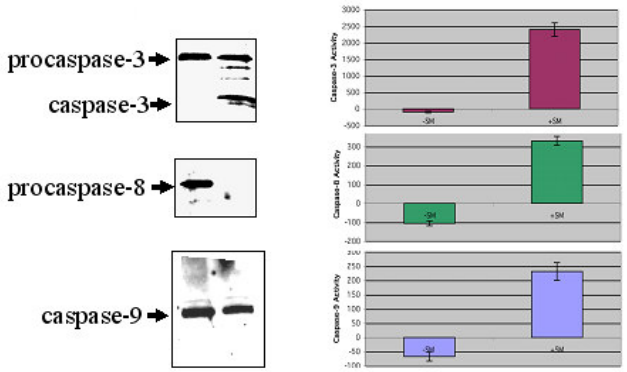

FIGURE 6. SM (0.3 mM, 16-18 hours) causes **c**aspase-3, caspase-8 and caspase-9 processing and activation in human small airway epithelial cells (SAEC). Cell extracts were used in these experiments. The substrates used for assays of caspase-9, caspase-8 and caspase-3 were AC-LEHD-AMC, AC-IETD-AMC and AC-DEVD-AMC, respectively. Assays of caspases and immunoblotting were as described in Materials and Methods.

CONCLUSIONS

- SM causes injury to the skin, the eyes, and the respiratory tract via epithelial damage due to basal cell apoptosis.
- SM (0.3 mM) causes apoptosis (caspase activation) in cultured human keratinocytes (skin model) and airway epithelial cells (respiratory tract model).
- The pathways of apoptosis due to SM in both skin and respiratory tract are the following:
  (a) Intrinsic mitochondrial pathway as indicated by the initiator caspase-9 activation
  (b) Extrinsic Fas (death receptor) pathway as indicated by the initiator caspase-8 activation
- SM also activates the executioner caspase-3, which is responsible for apoptosis via both the mitochondrial and the Fas pathways.
- Caspase-3 activation due to SM is prevented by the presence of the peptide caspase-3 specific inhibitor AC-DEVD-CHO (or the general caspase inhibitor Z-VAD-FMK) in culture medium of cells exposed to SM.
- These results indicate that both the mitochondrial and the Fas pathways of apoptosis are common features in skin as well as inhalation injury due to SM.
- Peptide caspase inhibitors may serve as prospective cutaneous therapeutics as well as respiratory therapeutics against SM injury.

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